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The *Env*-like Open Reading Frame of the Baculovirus-Integrated Retrotransposon TED Encodes a Retrovirus-like Envelope Protein

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TED is a 7.5-kbp member of the gypsy family of retrotransposons that was first identified by its integration within the baculovirus DNA genome. This lepidopteran (moth) transposon contains three retrovirus-like genes, including functional *gag* and *pol* that yield reverse transcriptase-containing virus-like particles. To identify and characterize the product(s) of the third *env*-like open reading frame, TED ORF3 was expressed in homologous lepidopteran cells by using a baculovirus vector, vENV. Immunoblots and immunoprecipitations with antiserum raised against a bacterial ORF3-fusion protein detected two ORF3-encoded proteins, $p68^{env}$ and $gp75^{env}$. On the basis of selective incorporation of [^{3}H]mannose and inhibition of modification by tunicamycin which blocks N-linked glycosylation, $gp75^{env}$ is a glycoprotein derived from core precursor $p68^{env}$. As predicted by the presence of a transmembrane domain near the carboxyl terminus, both $p68^{env}$ and $gp75^{env}$ were associated with heavy membranes of vENV-infected cells. Thus, TED ORF3 encodes a membrane glycoprotein with properties characteristic of retroviral *env* proteins. These data are consistent with the hypothesis that TED is an invertebrate retrovirus. Moreover, TED integration within the baculovirus genome provides an example of retroelement-mediated acquisition of host genes that may contribute to virus evolution.

INTRODUCTION

Transposable Element D (TED) is a 7510-bp retrotransposon that resides within the genome of the cabbage looper *Trichoplusia ni,* a nocturnal moth (Lepidoptera; Noctuidae). On the basis of sequence similarity and gene organization (Friesen and Nissen, 1990), TED is a member of the gypsy family of retrotransposons that bears a striking resemblance to the vertebrate retroviruses (for reviews, see Boeke and Corces, 1989; Friesen, 1993; Finnegan, 1994). Typical of the gypsy elements, TED is flanked by long terminal repeats (LTRs) with a U3-R-U5 retrovirus structure that directs the transcription of full-length RNAs representing potential templates for reverse transcription during transposition (Friesen and Nissen, 1990). The LTRs flank three open reading frames analogous in size and position to the retroviral *gag, pol,* and *env* genes (Fig. 1). Among the diverse classes of eukaryotic transposons, the presence of the third *env*-like ORF (ORF3) is unique to TED and other gypsy family members, including the *Drosophila melanogaster* elements gypsy, 17.6, 297, and tom (Marlor *et al.,* 1986; Saigo *et al.,* 1984; Inouye *et al.,* 1986; Tanda *et al.,* 1994). Since retrovirus *env* proteins mediate viral infectivity through host cell receptor recognition and fusion of viral and cellular membranes (recently reviewed by Weiss, 1993; Coffin, 1996), it was expected that the *env*-like ORF3 may function similarly for horizontal transmission of gypsy-related elements. Indeed, recent evidence suggests that gypsy virus-like particles are infectious in *Drosophila* and are associated with *env*-like proteins encoded by ORF3 (Kim *et al.,* 1994; Song *et al.,* 1994).

TED was first identified as a spontaneous insertion within the DNA genome (131 kbp) of *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV). As such, it represented the first retroid insertion within an animal virus (Miller and Miller, 1982). Transposition during infection of cultured *T. ni* cells generated TED-containing Ac*M*NPV mutant FP-D that was distinguished by its reduced production of occluded virus particles. $TED^{FP\text{-}D}$ integration disrupted a nonessential early gene (*p94*) and altered transcription within the region (Friesen *et al.,* 1986). A variety of mobile elements with diverse mechanisms of transposition have been identified within the Ac*M*NPV genome (Miller and Miller, 1982; Carstens, 1987; Wang *et al.,* 1989; Beames and Summers, 1990; Bauser *et al.,* 1996). Thus, as evidenced by the frequent insertion of lepidopteran transposons, the baculovirus genome has the unique capacity to accommodate host-derived DNA elements (reviewed by Blissard and Rohrmann, 1990; Friesen, 1993). Such events provide genetic diversity and may contribute to baculovirus evolution through the acquisition of new genes and regulatory signals.

The genetic organization of $TED^{FP\text{-}D}$ (Fig. 1) is representative of the approximately 50 copies of TED within the *T. ni* genome (Miller and Miller, 1982; Lerch and Friesen,

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1992). Recombinant baculovirus-mediated expression of TED *gag* and *pol* yields virus-like particles (VLP) (55 to 60 nm in diameter) that contain active reverse transcriptase (Lerch and Friesen, 1992). The major VLP capsid protein $p37^{gag}$ is derived by cleavage of precursor $Pr55^{gag}$ encoded by TED *gag.* TED *pol* encodes the aspartic protease responsible for cleavage of $Pr55^{gag}$ and processing of the *gag-pol* polyprotein that yields active reverse transcriptase (Hajek and Friesen, 1996). Thus, TED exhibits the properties of an active retroid element. Due to the presence of the third *env*-like ORF, an important issue is whether TED is an infectious lepidopteran retrovirus, an intracellular retrotransposon, or both.

Due to the expected requirement for an *env*-like protein in cell-to-cell transmission of virus-like particles, we have characterized the protein products of TED ORF3 as a first step in investigating the possibility that TED is an invertebrate retrovirus. By using a TED ORF3-expressing Ac*M*NPV vector (vENV) and polyclonal antiserum against bacterial ORF3-fusion protein, we report here that TED ORF3 encodes two related proteins, $p68^{env}$ and $gp75^{env}$. As demonstrated by using cultured lepidopteran cells, $gp75^{env}$ is a glycoprotein containing N-linked carbohydrates, including mannose. Both proteins were associated with cellular membranes as predicted by the presence of a hydrophobic transmembrane domain near their C terminus. Thus, TED encodes a membrane-bound glycoprotein with retroviral-like properties required for infectivity. These findings provide the opportunity to directly test cell-to-cell movement of TED and to further examine the evolutionary relationship between retroviruses and retrotransposons.

MATERIALS AND METHODS

Cells and viruses

Established lepidopteran (moth) cell lines *Spodoptera frugiperda* IPLB-SF21 (SF21) (Vaughn, 1977) and *Trichoplusia ni* BTI-TN-5B1-4 (TN High 5) (Wickham *et al.,* 1992) were propagated in TC100 growth medium (GIBCO Laboratories) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone Laboratories) and 2.6 mg/ml of tryptose broth. Viruses used included wild-type Ac*M*NPV (L-1 strain) (Lee, 1978) and an Ac*M*NPV *polyhedrin* deletion mutant (ΔPOLY), kindly provided by Lois Miller (University of Georgia).

Recombinant plasmids

The complete TED ORF3 was subcloned from plasmid pTED/P_3P_4 (Friesen and Nissen, 1990) by digestion with *Afl*III, end repair with Klenow fragment, and digestion with *Pvu*II. Blunt-end ligation of the resulting 1826-bp fragment into the *Sma*I site of pBluescript (KS) vector (Stratagene) generated plasmid pBS/env. To construct transplacement vector pAC700/env, the ORF3-containing *Bam*HI–*Kpn*I fragment of pBS/env was inserted into the corresponding sites of pAC700 (Invitrogen) such that the ATG start codon of *polyhedrin* was fused to Asp-Pro-Pro codons followed by TED ORF3. TrpE-*env* expression plasmid pATH22/env was generated by inserting the 1128-bp *Bam*HI–*Xba*I fragment of pAC700/env into the corresponding sites of pATH22 (Koerner *et al.,* 1991).

Construction of Ac*M*NPV recombinant vENV

Standard gene replacement methods (O'Reilly *et al.,* 1992) were used to generate vENV in which *polyhedrin* was replaced by TED ORF3 under control of the *polyhedrin* promoter. pAC700/env (10 μg) and wild-type Ac*M*NPV DNA (1 μg) were transfected into SF21 cells by using Lipofectin (Bethesda Research Laboratories) and virus progeny was harvested 4 days later. Occlusion-negative Ac*M*NPV recombinants were identified and plaque purified. Proper insertion of TED ORF3 was verified by restriction mapping of isolated viral DNA (data not shown).

Radiolabeling

Monolayers of SF21 or TN High 5 cells were inoculated with a multiplicity of infection (m.o.i.) of 10 or 20 plaque-forming units per cell, overlaid with fresh growth medium, and incubated at 27°. At the designated times, the growth medium was replaced with methionine-deficient Grace's medium-2% FBS containing 100 μCi/ml Trans ^{35}S-Label ($>$1000 Ci/mmol, methionine $>$ 70%, cysteine $<$ 15%; ICN Biomedicals, Inc.) or TC100-2% FBS containing 120 μCi/ml [^{3}H]mannose (27 Ci/mmol; DuPont NEN). After the indicated incubation period, the cells were dislodged, collected by low-speed centrifugation, washed with phosphate-buffered saline (PBS), pH 6.2, and lysed by boiling in 1% sodium dodecyl sulfate (SDS)–2.5% β-mercaptoethanol. Lysates were subjected to SDS–12.5% polyacrylamide gel electrophoresis (Laemmli, 1970) followed by fluorography.

Antiserum preparation

Polyclonal rabbit serum α-TrpE-env was generated against a TrpE–env fusion protein containing the N-terminal 376 amino acids encoded by TED ORF3. Fusion protein was overproduced in pATH22/env-containing *Escherichia coli* (strain JM83), excised from a stained polyacrylamide gel, and emulsified with an equal volume of Freund's complete adjuvant. A New Zealand white rabbit was intradermally injected with TrpE–env protein ($\sim$300 μg) and boosted three times ($\sim$300 μg each using Freund's incomplete adjuvant). Preimmune and immune serum were collected, heat-treated, and stored at $-70°$. Standard protocols were used for all steps (Harlow and Lane, 1988).

Immunoblot analysis

Cell extracts were subjected to SDS–10% polyacrylamide gel electrophoresis and transferred to nitrocellulose as described previously (Towbin *et al.,* 1979). The membranes were blocked with 3% BSA, 10 m*M* Tris–HCl (pH 7.4), 150 m*M* NaCl, and 0.05% Tween 20, washed, and incubated 1 hr with a 1:5000 dilution of α-TrpE–env. After washing, the membranes were incubated for 1 hr with a 1:5000 dilution of goat α-rabbit immunoglobulin (IgG; Pierce) conjugated to alkaline phosphatase. Color development was as described previously (Hershberger *et al.,* 1994). Monoclonal antibody AcV_5 (Hohmann and Faulkner, 1983) that recognizes Ac*M*NPV glycoprotein gp64 was kindly provided by Gary Blissard (Cornell University). AcV_5 was diluted 1:100 and used in combination with a 1:5000 dilution of goat α-mouse IgG (Jackson Labs) conjugated to alkaline phosphatase.

Immunoprecipitations

At the designated times after infection, [^{35}S]Met-Cys-labeled cells were collected by low-speed centrifugation, washed with PBS (pH 6.2), and suspended in ice-cold NP-40 buffer (1% NP-40, 150 m*M* NaCl, 50 m*M* Tris (pH 8.0)) at a final concentration of 10^6 cells/ml. After 30 min on ice, the lysates were clarified by centrifugation (10,000 *g*) and the supernatant was stored at −80°. For immunoprecipitation (Anderson and Blobel, 1983), lysates corresponding to 3×10^5 cell equivalents were diluted to 1 ml with 1.25% Triton X-100, 190 m*M* NaCl, 60 m*M* Tris (pH 7.4), 6 m*M* EDTA, 2 m*M* benzamidine, and mixed with α-TrpE–env (20 μl). After 12 to 16 hr, immune complexes were collected with protein A–Sepharose beads, washed with 0.1% Triton X-100, 0.02% SDS, 150 m*M* NaCl, 5 m*M* Tris (pH 7.4), 5 m*M* EDTA, 2 m*M* benzamidine, and eluted by boiling in 1% SDS–2.5% β-mercaptoethanol. The supernatant was subjected to SDS–12.5% polyacrylamide gel electrophoresis and fluorography.

Subcellular fractionation

Infected TN High 5 cells were washed in PBS, pH 6.2, suspended in 10 m*M* Tris (pH 7.5), 5 m*M* $MgCl_2$, 1 m*M* phenylmethylsulfonylfluoride (PMSF), and disrupted by Dounce homogenization as described previously (Hershberger *et al.,* 1994). After stabilization of the nuclei by adjustment to 140 m*M* NaCl and 40 m*M* KCl, the extract was separated by centrifugation (300 *g,* 5 min) into an S1 fraction and a nuclear (N1) pellet. The N1 pellet was suspended in nuclei buffer (10 m*M* Tris (pH 7.5), 5 m*M* $MgCl_2$, 140 m*M* NaCl, 40 m*M* KCl) containing 0.25 *M* sucrose and pelleted (44,000 *g*) through a 1.6 *M* sucrose cushion. The resulting nuclear pellet (N2) was suspended in nuclei buffer and incubated for 30 min in 1% Triton X-100. The detergent-treated nuclei (N3) were collected by centrifugation (300 *g,* 5 min). After adjustment to 5 m*M* EDTA, the S1 fraction (see above) was clarified by centrifugation (10,000 *g,* 10 min) to obtain the cytoplasmic (C) fraction. The resulting pellet containing heavy membranes (HM) was washed and suspended in 10 m*M* Tris (pH 7.5), 140 m*M* NaCl, 40 m*M* KCl, and 1 m*M* PMSF. Samples of each fraction were boiled in 1% SDS–2.5% β-mercaptoethanol prior to immunoblot analysis.

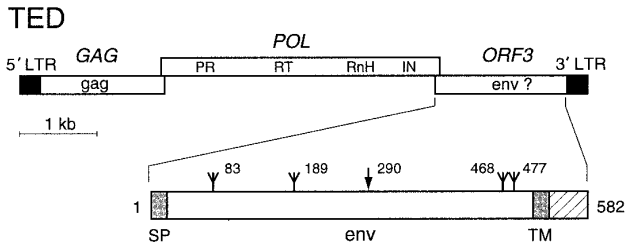

FIG. 1. Genetic organization of the retrotransposon TED. The retroviral-like *gag, pol,* and *env*-like ORFs (open boxes) of TED are flanked by 5′ and 3′ LTRs. The highly conserved protease (PR), reverse transcriptase (RT), RNase H (RnH), and integrase (IN) domains within *pol* are indicated. The predicted protein product of TED ORF3 is depicted (below) with potential glycosylation (Y) sites (residues 83, 189, 468, and 477) and the protease cleavage site (arrow) at residue 290. Predicted hydrophobic regions (shaded boxes) corresponding to the signal peptide (SP) and transmembrane (TM) domain, respectively, and the hydrophilic C-terminal tail (crosshatched) are indicated.

Image processing

Autoradiograms and immunoblots were scanned at a resolution of 300 dpi by using a Microtek Scanmaker III equipped with a transparency adapter. The resulting files were printed from Adobe Photoshop 2.5 by using a Tektronix Phaser IISDX dye-sublimation printer.

RESULTS

Predicted properties of the TED *env*-like protein

Although retroviral *env* proteins bear little sequence similarity, several general features are shared by these membrane glycoproteins, including an N-terminal signal peptide, a transmembrane domain followed by a C-terminal hydrophilic tail, and an endopeptidase cleavage site (reviewed by Hunter and Swanstrom, 1990; Weiss, 1993; Coffin, 1996). The predicted 67-kDa protein encoded by TED ORF3 (Fig. 1) contains a potential hydrophobic signal sequence at its N terminus (residues 1 to 20) and a transmembrane domain (residues 514 to 532) near the C terminus that could function as a membrane anchor. The transmembrane domain of the predicted TED *env*-like protein is followed by a highly charged, hydrophilic tail (residues 533 to 582). In addition, there are four consensus N-linked glycosylation motifs at residues 83, 189, 468, and 477 (Fig. 1). Last, the TED *env*-like protein has several potential protease cleavage sites similar to the retrovirus *env* cleavage motif Arg/Lys-X-Lys-Arg. The potential site near ORF3 residue 290 (Ile-Asp-Lys-Arg) is at

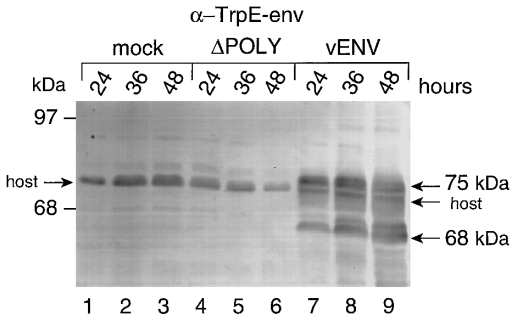

FIG. 2. Immunoblot analysis of vENV-specific proteins. TN High 5 cells were harvested and lysed with SDS at the indicated times after mock-infection (mock) or infection with Ac*M*NPV polyhedrin-deletion mutant (ΔPOLY) or recombinant vENV (m.o.i.s of 10). Lysates (2×10^5 cell equivalents per lane) were subjected to polyacrylamide gel electrophoresis and immunoblot analysis by using α-TrpE-env serum. Molecular weight standards (sizes in kilodaltons) and TED *env*-like proteins (arrows) are shown.

an analogous position for a viral *env* protein and is followed by a stretch of hydrophobic residues that may function as the required fusion domain (Gallaher *et al.,* 1989).

Identification of TED ORF3-encoded proteins

To identify and characterize the *env*-like protein(s) of TED, we constructed Ac*M*NPV recombinant vENV in which TED$^{FP\text{-}D}$ ORF3 was placed under control of the very late *polyhedrin* promoter and substituted for the *polyhedrin* gene. In retroviruses, *env* is expressed from a mRNA derived by splicing of the full-length genomic RNA (reviewed by Coffin, 1996). The resulting *env* mRNA usually contains 5′ leader sequences from upstream or within the *gag* ORF that are spliced to the *env* ORF and subsequently terminate within the 3′ LTR. Nucleotide sequence analysis indicated that the *pol–env* junction of TED$^{FP\text{-}D}$ ORF3 was identical to *T. ni* genomic copies of TED, including the absence of an ATG start codon (data not shown). Thus, it was expected that the ORF3 used here was representative of endogenous TED elements, in particular at potential splice acceptor sites on the 5′ side of ORF3. Since TED ORF3 lacks an initiating ATG, it was expressed as an in-frame fusion which extended the 5′ end of the ORF by Met-Asp-Pro-Pro codons. These N-terminal residues were not expected to affect signal sequence function due to their distance from the predicted cleavage site (von Heijne, 1983).

TED ORF3-specific proteins were identified in cultured lepidopteran cells infected with recombinant vENV by using immunoblot analysis. To this end, polyclonal rabbit antiserum (α-TrpE–env) was generated against an *E. coli*-derived TrpE–env fusion protein containing the N-terminal 376 amino acids encoded by TED$^{FP\text{-}D}$ ORF3. Two prominent proteins with molecular masses of 68 and 75 kDa were detected by α-TrpE–env serum in vENV-infected TN High 5 cells (Fig. 2). Both proteins accumulated from 24 to 48 hr after infection (lanes 7 to 9), consistent with *polyhedrin* promoter-directed expression. α-TrpE–env also detected a 74-kDa protein in mock-infected and ΔPOLY-infected TN High 5 (Fig. 2) and SF21 cells (data not shown). Consistent with its host origin, the 74-kDa protein accumulated in mock-infected cells (lanes 1 to 3) but decreased in ΔPOLY-infected cells (lanes 4 to 6). Preimmune serum failed to recognize the 74-kDa host protein or TED ORF3-encoded proteins (data not shown).

The 68- and 75-kDa TED proteins were specifically detected in [^{35}S]Met-Cys-labeled lysates from lepidopteran cells infected with vENV. Both proteins were radiolabeled during infection of TN High 5 cells (Fig. 3A) and SF21 cells (Fig. 3B). The most prominent virus-specific protein in this size range was Ac*M*NPV glycoprotein gp64, the major envelope fusion protein (EFP) that was detected in both cell lines after infection with wild-type, ΔPOLY, and vENV viruses (lanes 1 to 6). On the basis of relative mobility with respect to gp64 and molecular weight standards, the TED ORF3 proteins exhibited similar sizes when synthesized in either cell line.

The identity of the TED ORF3-specific proteins was confirmed by immunoprecipitation (Fig. 4A). Both the 68- and 75-kDa proteins were immunoprecipitated with α-TrpE–env from [^{35}S]Met-Cys-labeled extracts of vENV-infected TN High 5 cells (lanes 3 and 4). In contrast, α-TrpE–env failed to precipitate radiolabeled proteins from ΔPOLY-infected cells (Fig. 4A, lanes 1 and 2), including the cross-reactive 74-kDa host protein detected by immunoblots (Fig. 2). Less abundant proteins, ranging in molecular mass from 35 to 50 kDa, were also

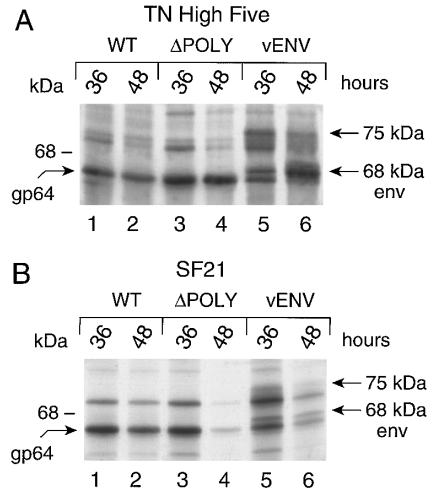

FIG. 3. Identification of radiolabeled vENV-specific proteins. TN High 5 (A) and SF21 (B) cells were infected with wild-type (WT) Ac*M*NPV, ΔPOLY, or vENV (m.o.i. of 10 and 20 for TN High5 and SF21 cells, respectively) and radiolabeled with [^{35}S]Met-Cys for a 6-hr period prior to the indicated times after infection. Cells were harvested, lysed with SDS, and subjected to polyacrylamide gel electrophoresis and fluorography. Molecular weight standards (sizes in kilodaltons), TED *env*-specific proteins (arrows), and gp64 are indicated.

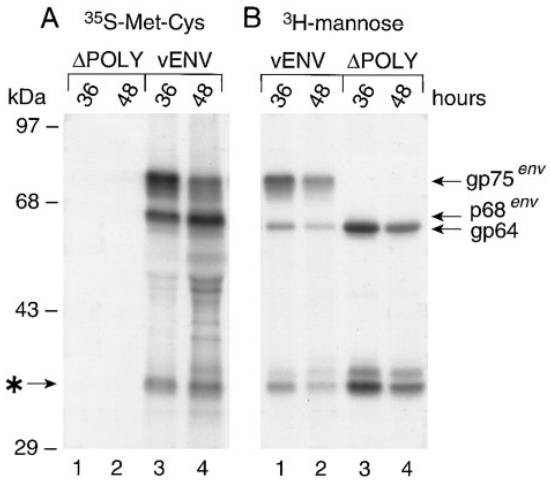

FIG. 4. Comparison of [35S]Met-Cys-labeled and [3H]mannose-labeled TED ORF3-encoded proteins. (A) Immunoprecipitation. TN High 5 cells were radiolabeled with [35S]Met-Cys for 6 hr prior to lysis at 36 and 48 hr after infection with ΔPOLY or vENV. The resulting radiolabeled extracts (3×10^5 cell equivalents) were immunoprecipitated with α-TrpE-env serum. The potential *env*-like proteolytic cleavage product (*) is indicated at the left. (B) Radiolabeling with [3H]mannose. TN High 5 cells were radiolabeled with [3H]mannose for 12 hr prior to lysis at 36 and 48 hr after infection with vENV or ΔPOLY. Protein samples were subjected to polyacrylamide gel electrophoresis and fluorography. Molecular weight standards (sizes in kilodaltons), TED *env*-specific proteins, and Ac*M*NPV gp64 EFP are shown.

immunoprecipitated from vENV-infected TN High 5 cell extracts. The most prominent of these was a 34-kDa protein (Fig. 4A (*)). Adsorption with preimmune serum failed to reduce the level of these proteins (data not shown) and indicated that they were specifically recognized by α-TrpE–env. As such, the 34-kDa ORF3-specific protein represents a potential proteolytic cleavage product of the larger ORF3 proteins. Collectively, these data demonstrated that TED ORF3 encodes two or more related polypeptides, the most abundant of which are the 68- and 75-kDa proteins.

TED ORF3 proteins are glycosylated

The 75-kDa TED ORF3-specific protein was significantly larger than its predicted mass of 67 kDa. Moreover, since retroviral envelope proteins are glycosylated, we hypothesized that the 75-kDa protein represented a glycosylated form of the 68-kDa core protein. To test this possibility, we first determined whether the 75-kDa protein could be selectively radiolabeled with [3H]-mannose in vENV-infected cells. Electrophoretic analysis of [3H]mannose-labeled lysates of TN High 5 cells late after vENV infection detected the 75-kDa protein, but not the 68-kDa protein (Fig. 4B, lanes 1 and 2). Direct comparison of the electrophoretic mobility of the [3H]mannose-labeled proteins with that of [35S]Met-Cys-labeled proteins immunoprecipitated with α-TrpE–env (Fig. 4A, lanes 3 and 4) confirmed the identity of the 75-kDa protein. As expected, Ac*M*NPV glycoproteins were also radiolabeled with [3H]mannose, including gp64 EFP and smaller proteins with molecular masses of 35 and 36 kDa. Due to the similar mobilities of these viral proteins, it was not possible to verify [3H]mannose-labeling of the 34-kDa putative ORF3 cleavage product (*).

Tunicamycin inhibition of N-linked glycosylation in vENV-infected cells confirmed that the 75-kDa TED ORF3 polypeptide is a glycoprotein (Fig. 5). In the presence of increasing concentrations of tunicamycin, accumulation of the 75-kDa protein decreased, whereas the level of the 68-kDa protein was unaffected (lanes 5 to 8). All concentrations of tunicamycin failed to affect the 74-kDa host protein (lanes 1 to 4), suggesting that this protein is not N-linked glycosylated. In contrast, tunicamycin dramatically inhibited glycosylation of gp64 EFP, as indicated by the sharp increase in electrophoretic mobility upon drug treatment in immunoblot analysis using α-gp64 serum (lanes 9 to 12). These findings indicated that the 75-kDa TED ORF3 protein contains N-linked carbohydrates, including mannose. Since carbohydrate groups have a typical mass of 1.5 to 2 kDa (Kornfield and Kornfield, 1985), the addition of N-linked carbohydrate groups at the four predicted glycosylation sites of the 68-kDa protein would account for the size of the 75-kDa protein. On the basis of these data, we hereafter refer to the 75-kDa TED ORF3-encoded protein as gp75^{env} and to the unmodified form as p68^{env}.

TED proteins gp75^{env} and p68^{env} are associated with membranes

By virtue of their transmembrane domain, retroviral envelope proteins localize to the plasma membrane of the infected host cell where they associate with virus particles during the budding process (reviewed by Coffin, 1996). To determine whether TED gp75^{env} and p68^{env} also

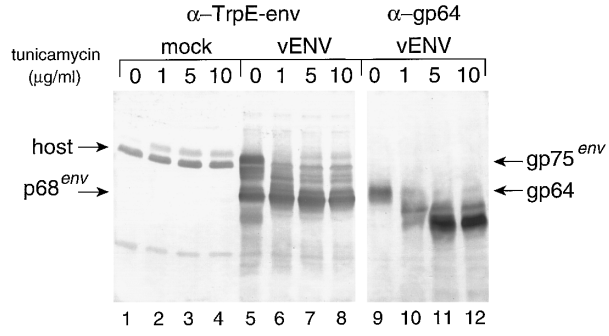

FIG. 5. Effect of tunicamycin on TED *env*-specific proteins. TN High 5 cells were incubated with the indicated concentrations (μg/ml) of tunicamycin in growth medium beginning 4 hr after mock-infection (lanes 1 to 4) or infection with vENV (lanes 5 to 12). Total cell proteins were prepared by SDS-lysis 36 hr after infection and subjected to immunoblot analysis (10^5 cell equivalents per lane) by using α-TrpE-env (lanes 1 to 8) or α-gp64 AcV_5 (lanes 9 to 12). Host, gp64, and TED *env*-specific proteins are indicated.

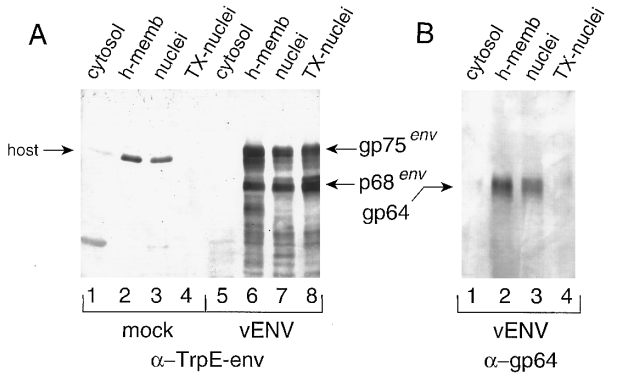

FIG. 6. Subcellular distribution of TED *env*-specific proteins. Mock- or vENV-infected TN High 5 cells were homogenized and subjected to differential centrifugation. Subcellular fractions of cytosol (lanes 1 and 5), heavy membranes (lanes 2 and 6), intact nuclei (lanes 3 and 7), or Triton X-100 extracted nuclei (lanes 4 and 8) were subjected to SDS–polyacrylamide gel electrophoresis and immunoblot analysis by using (A) α-TrpE-env or (B) α-gp64 AcV_5. Host, gp64, and TED *env*-specific proteins are indicated.

associate with cellular membranes, vENV-infected TN High 5 cells were homogenized and fractionated by differential centrifugation. Both $gp75^{env}$ and $p68^{env}$ were detected in fractions containing heavy membranes and intact nuclei, but not the cytosol (10,000 *g* supernatant) (Fig. 6A, lanes 5 to 7). These proteins cofractionated with membrane-associated gp64 EFP that was present in heavy membrane and perinuclear fractions, but not the cytosol (Fig. 6B). However, unlike gp64 EFP, $gp75^{env}$ and $p68^{env}$ associated with nuclei even after solubilization of the perinuclear membrane with Triton X-100 (Figs. 6A and 6B). Although these TED *env*-like proteins may be tightly associated with the nucleus, protein–protein aggregation of *env*-specific proteins could account for cofractionation with detergent-extracted nuclei. Consistent with protein aggregation, $p68^{env}$ and $gp75^{env}$ sedimented to the most dense fractions in sucrose density gradients of extracts from vENV-infected TN High 5 cells (data not shown). The 74-kDa host protein recognized by α-TrpE–env was also associated with heavy membrane and intact nuclear fractions, but was extracted by Triton X-100 (Fig. 6A, lanes 1 to 4). In summary, TED $gp75^{env}$ and $p68^{env}$ associated with cellular membranes, as expected for retroviral *env* proteins.

DISCUSSION

Env-like properties of TED ORF3 proteins

By overexpressing $TED^{FP\text{-}D}$ ORF3 using an Ac*M*NPV recombinant, we have identified two related proteins, $p68^{env}$ and $gp75^{env}$, that are encoded by this *env*-like gene and exhibit many characteristics of retrovirus envelope proteins. A major advantage to this approach was the higher level of TED ORF3 expression in homologous lepidopteran cells which facilitated characterization of the processed *env*-like gene products. Due to low levels of endogenous TED expression, we have not yet detected TED-specific proteins in cultured (uninfected) *T. ni* cells (data not shown).

Both $p68^{env}$ and $gp75^{env}$ were specifically recognized by antiserum generated against a bacterial TrpE–env fusion protein (Figs. 2 and 4), verifying that these proteins are encoded by TED ORF3. As indicated by radiolabeling with [^{3}H]mannose and tunicamycin inhibition (Figs. 4 and 5), TED $gp75^{env}$ is modified by N-linked glycosylation. The apparent lack of carbohydrate modification of $p68^{env}$ and the finding that its size approximates that predicted by nucleotide sequence (67 kDa) suggest that $p68^{env}$ is the nonglycosylated precursor to $gp75^{env}$. Since the size of both TED proteins was similar when synthesized in different cell lines (Figs. 3A and 3B), protein modification was not cell-line specific.

As determined by subcellular fractionation, TED $p68^{env}$ and $gp75^{env}$ were associated with the same heavy membranes as Ac*M*NPV gp64 EFP (Fig. 6). This membrane association combined with the presence of a putative signal peptide and transmembrane domain (Fig. 1) indicated that $p68^{env}$ and $gp75^{env}$ are membrane proteins, as expected for a retrovirus *env* protein. However, cofractionation of the TED proteins with detergent-extracted nuclei was unexpected (Fig. 6). The presence of TED *env* protein here could be due to nonspecific protein aggregation resulting from high level synthesis late in infection when the efficiency of the host glycosylation and secretory pathway is reduced (Jarvis and Summers, 1989). Nonetheless, we have not formally ruled out the interesting possibility that $p68^{env}$ and $gp75^{env}$ are targeted to the nucleus where membranes are synthesized *de novo* during maturation of occluded Ac*M*NPV particles (Stoltz *et al.,* 1973).

In a process that is required for infectivity, retroviral *env* proteins are cleaved at dibasic motifs (Arg/Lys-X-Lys-Arg) by a host endopeptidase to generate surface and transmembrane peptides (reviewed by Coffin, 1996). Cleavage of TED *env* proteins at a putative protease site (Ile-Asp-Lys-Arg) near residue 290 would generate two ~34-kDa peptides. Whereas immunoblots detected multiple smaller proteins, immunoprecipitations revealed a prominent 34-kDa protein synthesized in infected cells expressing TED ORF3 (Fig. 4A). Although the origin of this TED ORF3-specific protein needs to be determined, its size is consistent with endopeptidase processing of the TED *env*-like proteins.

Are the gypsy retrotransposons invertebrate retroviruses?

Current theories contend that retrotransposons are the evolutionary predecessors of the retroviruses, noninfectious retrovirus derivatives, or both (Temin, 1980; Xiong and Eickbush, 1990; Finnegan, 1994; Löwer *et al.,* 1996).

The gypsy retrotransposons are the closest retrovirus relatives due to the high sequence similarity within *pol* and the presence of a third *env*-like ORF. TED is the first nondipteran retrotransposon (Order Lepidoptera) demonstrated to encode a retrovirus *env*-like glycoprotein. In *Drosophila,* gypsy and tom encode unrelated *env*-like proteins (Tanda *et al.,* 1994; Song *et al.,* 1994). Further analysis of the retrovirus-like nature of TED gp75^{env} must include demonstration of expression by splicing of full-length genomic RNA as is the case for *env* of gypsy and tom (Pelisson *et al.,* 1994; Tanda *et al.,* 1994). Moreover, gp75^{env} must associate with a membrane envelope of TED VLP during budding to mediate infectivity. Preliminary efforts to detect such association by using very late baculovirus expression vectors have been inconclusive (data not shown) and may require TED *env* expression earlier in infection or in stably transfected cells. Nonetheless, current evidence suggests that the gypsy *env* protein associates with VLP and that such retrovirus-like particles are infectious as judged by their capacity to confer a high level of gypsy insertion activity in *Drosophila* strains upon injection or feeding (Kim *et al.,* 1994; Song *et al.,* 1994). Formal demonstration that members of the gypsy family can exist as infectious viruses requires genomic integration of genetically tagged proviruses by a process that is susceptible to extracellular neutralization. Progress toward determining the retrovirus nature of TED in particular is expected to contribute to the development of useful vectors for stable transformation of nondipteran insects, including the Lepidoptera for which limited transformation systems are available (Handler and O'Brochta, 1991).

Host transposon-mediated evolution of the baculoviruses

Due in part to the capacity of the baculovirus genome to accommodate foreign DNA, diverse classes of lepidopteran host transposons have been identified as viral DNA insertions (reviewed by Friesen, 1993). Since host transposons have been documented to disrupt viral genes and alter viral regulatory sequences, such insertions have a significant impact on the viral genome and are thus expected to contribute to baculovirus evolution. In the case of TED$^{FP\text{-}D}$, Ac*M*NPV integration contributed new transcriptional control sequences (LTRs) and functional capsid (*gag*) protein, aspartic protease, and reverse transcriptase genes (Friesen *et al.,* 1986; Lerch and Friesen, 1992). As reported here, TED insertion also provides the genetic information for a membrane-associated glycoprotein (gp75^{env}). Due to the presence of multiple late viral promoter motifs (ATAAG) within TED near the start of ORF3, the synthesis of gp75^{env} or a modification thereof late in infection is likely. It is therefore tempting to speculate that the expression of TED or related retrotransposon *env*-like genes could contribute to baculovirus host range.

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